Synergistic interaction between wavelength of light and concentration of H202 in bactericidal activity of photolysis of H202

博士論文

Synergistic interaction between wavelength of light and concentration of H_2O_2 in bactericidal activity of photolysis of H_2O_2

（過酸化水素光分解殺菌技術における光の波長と過酸化水素濃度の相乗作用）

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Content

Abstract

The present study aimed to evaluate the interaction between wavelength of light in the range of ultra violet A-visible and concentration of H_2O_2 in the reaction of photolysis of H_2O_2 from the point of view of hydroxyl radical (·OH) generation and the bactericidal activity. LEDs emitting the light at wavelengths of 365, 385, 400 and 465 nm were used at an irradiance of 1000 mW/cm^2. H_2O_2 was used at the final concentrations of 0, 250, 500, and 1000 mM. Quantitative analysis of ·OH generated by the LED irradiation of H_2O_2 were performed using an electron spin resonance-spin trapping technique. In a bactericidal assay, a bacterial suspension of *Staphylococcus aureus* prepared in sterile physiological saline was irradiated with the LEDs. The bactericidal activity under each test condition was evaluated by viable counts. When H_2O_2 was irradiated with the LEDs, ·OH was generated and bacteria were killed dependently on the concentration of H_2O_2 and the wavelength of LED. The two-way analysis of variance revealed that the wavelength, the H_2O_2 concentration and their interaction significantly affected the yield of ·OH and the bactericidal activity of the photolysis of H_2O_2. Therefore, it is suggested that bactericidal activity of photolysis of H_2O_2 could be enhanced by controlling the wavelength and the concentration of H_2O_2, which may contributes to shortening the treatment time and/or to reducing the concentration of H_2O_2.

Introduction

A disinfection treatment utilizing hydroxyl radical (·OH) generated by photolysis of hydrogen peroxide (H_2O_2) has been developed in our laboratory (1, 2) (Figure 1). The reactive oxygen species including ·OH kill bacteria by oxidation of cellular components (3). Of reactive oxygen species, ·OH has the highest reactivity (4), which in turn exerts the highest bactericidal effect. *In vitro* studies demonstrated that *Staphylococcus aureus*, *Streptococcus mutans*, *Enterococcus faecalis*, and *Aggregatibacter actinomycetemcomitans* were killed with a >5-log reduction of viable counts within 3 min when the bacterial suspension was treated with ·OH generated by photolysis of 1000 mM H_2O_2 with blue light irradiation (wavelength: 405 nm) (1). In addition to the *in vitro* test, an *in vivo* antibacterial effect of the disinfection system based on photolysis of H_2O_2 was proven effective in a rat model of superficial *S. aureus* infection (5). Therefore, it is expected that the disinfection system is applied as a novel topical treatment for superficial infectious diseases such as periodontitis (6).

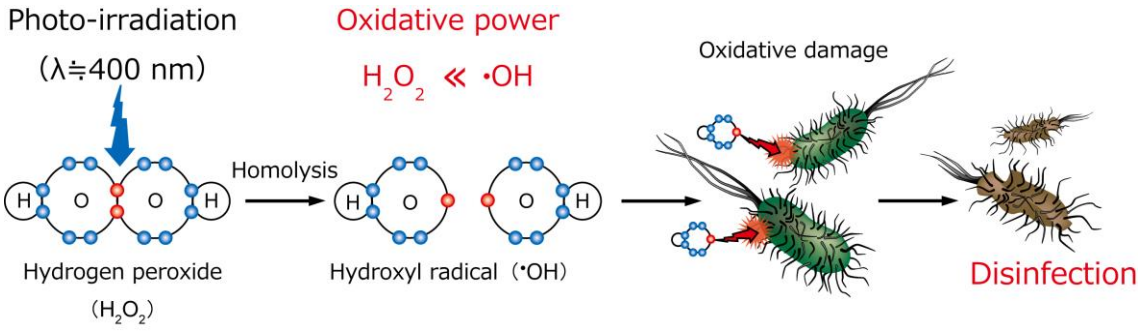

Figure 1. Mechanism of disinfection by photolysis of hydrogen peroxide

As for the safety aspects of the disinfection system, a literature review concluded that there would seem to be little or no risk of mutagenicity and carcinogenicity in using the ·OH as a disinfectant for short-time treatment (7). In addition, the previous study in our laboratory demonstrated that topical treatment with the photolysis of 1000 mM H_2O_2 had no detrimental effect on the oral mucosa and the healing process of full thickness skin wounds in rats (8). The 1000 mM H_2O_2 corresponds to approximately 3 %, which is used as a topical antiseptic for skin and oral mucosa. In Japan, 2.5-3.5% H_2O_2 known as Oxydol is approved by a regulating authority as an antimicrobial for external use. A subcommittee of the US Food and Drug Administration concluded that H_2O_2 is safe at concentrations of up to 3% (9). Nonetheless, H_2O_2 might cause adverse effects dependently on the concentration when applied in the oral cavity (10-12). Hence, a low concentration of H_2O_2 is preferable in the disinfection system based on photolysis of

H_2O_2 with respect to residual toxicity.

Regarding the light source, a laser or an LED with a wavelength of around 400 nm was used in the previous studies in our laboratory to avoid the possible adverse effect of ultraviolet (UV) light against normal tissue though UV would be advantageous in terms of bactericidal effect due to its potent ability to photolyze H_2O_2 (13). UV is defined as an electromagnetic wave with a wavelength of <400 nm (Figure 2). In addition, UV is subdivided into UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm) according to the wavelength (14). Since the energy of photon is expressed as $E=hc/\lambda$, where E is the energy, h is Planck's constant, c is the speed of light, and λ is the wavelength, the shorter the wavelength is the higher the energy of photon becomes (15). Accordingly, the cytotoxic effect caused by UV irradiation increases with the shortening of wavelength. It is well-known that UVC and UVB damages DNA and proteins though such effect of UVA is weak (16, 17). Besides the wavelength, the radiation intensity and the exposure time are also the factors influencing the harmful effect of UV irradiation. Since the disinfection treatment utilizing photolysis of H_2O_2 would be completed within a short-time (about 3 min), UV light, especially UVA, may be utilized to accelerate the reaction of photolysis for enhanced bactericidal activity without adverse effect. Indeed,

UVA is applied to a phototherapy in the treatment of skin disease and even to a “sunbed” (15, 18-20). If the bactericidal activity of the disinfection system based on the photolysis of H_2O_2 is enhanced by using UVA, the treatment time might be shortened and the concentration of H_2O_2 used in the disinfection system might also be reduced, resulting in a decrease in residual toxicity of H_2O_2. Thus, to achieve the application of UVA to the disinfection system it is necessary to obtain basic knowledge of bactericidal activity of photolysis of H_2O_2 when UVA is used as a light source.

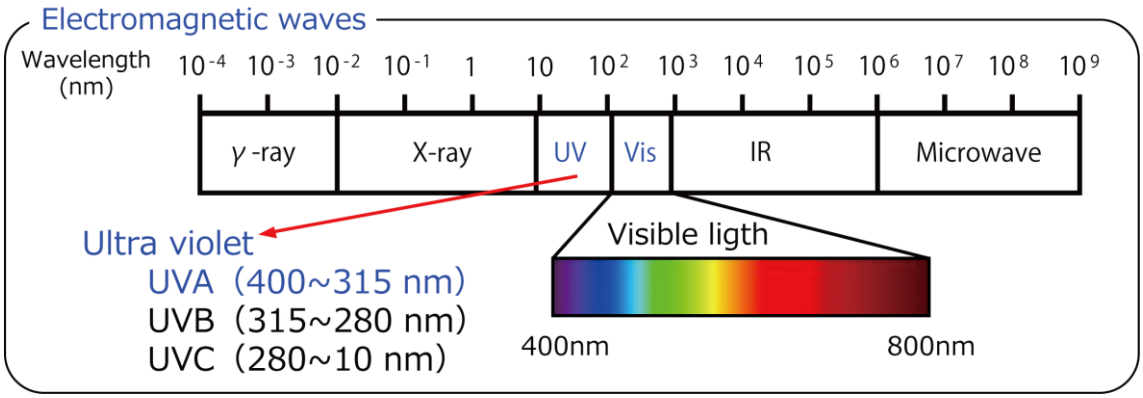

Figure 2. Classification of electromagnetic waves

The purpose of the present study was, therefore, to evaluate the interaction between wavelength of light in the range of UVA-Visible and concentration of H_2O_2 from the point of view of ·OH generation and the bactericidal activity.

Materials and Methods

Reagents. Reagents were purchased from the following sources: H_2O_2 from Santoku Chemical Industries (Tokyo, Japan); 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) from Labotec (Tokyo, Japan); 4-hydroxy-2,2,6,6-tetramethylpiperidine *N*-oxyl (TEMPOL) from Sigma Aldrich (St. Louis, MO). All other reagents used were of analytical grade.

Light source. An LED spot curing device (OmniCure LX400+, Lumen Dynamics Group, Ontario, Canada) with the specific heads emitting the light at wavelengths of 365, 385 and 400 nm was used (Figure 3). In addition, a dental LED light curing unit (G-Light Prima-II, GC, Tokyo, Japan) with a wavelength of 465 nm were also used (Figure 3). The output powers of the LEDs were measured using a power meter (FieldMate, Coherent, Santa Clara, CA), and the irradiance (mW/cm^2) was calculated by dividing the output power (mW) by the irradiation field size (cm^2). The output power of each LED was adjusted so that the LEDs could be used at the same irradiance (1000 mW/cm^2). The peak wavelength of each LED was measured using an illuminance spectrophotometer (CL-500A, Konica Minolta, Tokyo, Japan).

Figure 3. LED devices used in the present study

(Photos from the manufacturer's website).

Electron spin resonance (ESR) analysis of ·OH. Quantitative analysis of ·OH generated by photolysis of H_2O_2 was performed using an ESR-spin trapping technique according to the previous study in our laboratory (21). Since the life-time of ·OH was very short (< 10 ns) (4), the ·OH was firstly trapped by a spin trap reagent, DMPO, and then the ·OH trapped by DMPO, *i.e.* DMPO-OH, was quantitatively analyzed. In this assay, 500, 1000 and 2000 mM H_2O_2 prepared by diluting 31% (w/v) H_2O_2 with pure water was mixed with DMPO in a well of microplate to reach final concentrations of 250, 500 and 1000 mM for H_2O_2 and 300 mM for DMPO. Then, the sample was exposed to the LED irradiation at wavelengths of 365, 385, 400, and 465 nm for 0, 5, 10, and 15 s. After irradiation, the sample was transferred to a quartz cell for ESR spectrometry, and the ESR spectrum was recorded on an X-band ESR spectrometer

(JES-FA-100; JEOL, Tokyo, Japan). The measurement conditions for ESR were as follows: field sweep, 331.41–341.41 mT; field modulation frequency, 100 kHz; field modulation width, 0.05 mT; amplitude, 80; sweep time, 2 min; time constant, 0.03 s; microwave frequency, 9.420 GHz; and microwave power, 1 mW. TEMPOL (20 μM) was used as a standard to calculate the concentration of spin-trapped radicals, and the ESR spectrum of manganese (Mn^{2+}) held in the ESR cavity was used as an internal standard. The concentration of DMPO-OH was determined using Digital Data Processing (JEOL). In addition, the yield of DMPO-OH generated by the LED irradiation of pure water and autolysis of H_2O_2 without the LED irradiation were evaluated. All tests were performed in triplicate.

Bactericidal assay. *Staphylococcus aureus* JCM 2413 purchased from the Japan Collection of Microorganisms, RIKEN BioResource Center (Wako, Japan) was used. A bacterial suspension was prepared in sterile physiological saline from a culture grown on brain heart infusion (BHI) agar (Oxoid, Hampshire, UK), and the inoculum size was adjusted by using a colorimeter (WPA CO7500 colorimeter, Biochrom, Cambridge, UK). In a well of microplate, 100 μL of H_2O_2 or pure water was mixed with 100 μL of the bacterial suspension to reach final concentrations of 0, 250, 500 and 1000 mM for

H_2O_2 and approximately 3×10^7 colony forming units (CFU)/mL for the bacteria. Then, the sample was exposed to the LED irradiation at a wavelength of 365, 385, 400 or 465 nm for 1 min. After irradiation, 50 μL of the sample was mixed with an equal volume of sterile catalase solution (5000 U/mL) to terminate the bactericidal effect of remaining H_2O_2. A 10-fold serial dilution of the mixture was then prepared using sterile physiological saline and 10 μL of the diluted solution was seeded onto BHI agar plate. The agar plates were incubated aerobically at 37℃ for over 15 h. After that, the colonies grown on the agar plate were counted to determine the CFU/mL. In addition, a bacterial suspension that was kept for 1 min in a dark box instead of being irradiated was subjected to the same procedure. The bacterial initial count (inoculum size) in each assay was also evaluated by viable counting method using the same procedure. All tests were performed in sexuplicate.

Statistical analyses. Statistical significance ($p<0.05$) in the CFU/mL obtained in the bactericidal test was assessed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer honestly significant difference multiple comparison test. The analysis was performed following logarithmic conversion of CFU. When colony was not detected, the value of the detection limit (10^2 CFU/mL) was used for the statistical

analysis. Two-way ANOVA was also performed to determine if the yield of ·OH during LED irradiation for 15 s and the bactericidal activity of photolysis of H_2O_2 are interactively affected by the wavelength of light and the concentration of H_2O_2.

Results

Light source. The optical spectra of LEDs used in the present study are shown in Figure 4. The peaks of the LEDs were observed at 367, 384, 402 and 464 nm which are in accordance with manufacturer's data. The full width at half maximum (FWHM) was 15 nm for LEDs of OmniCure LX400+ irrespective of the wavelength and 26 nm for G-Light Prima-II. In addition, G-Light Prima-II had another peak at 400 nm though the radiation intensity was less than one tenth of the main peak at 464 nm.

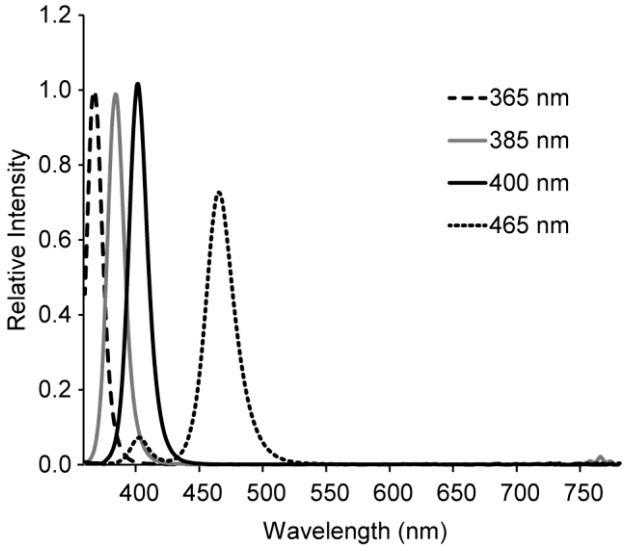

Figure 4. Spectra of LEDs used in the present study

ESR analysis of ·OH. When H_2O_2 was irradiated with the LEDs, DMPO-OH (a_N=a_H=1.49 mT, in which a_N and a_H are the hyperfine coupling constants arising from nitrogen and hydrogen atoms, respectively, in the structure of DMPO-OH(22)) was

detected (Figure 5). The yield of DMPO-OH depended on the concentration of H_2O_2, the wavelength of LED and the irradiation time (Figure 6). The shorter the wavelength was and the higher the concentration was, the more DMPO-OH was generated. It was found that even the LED irradiation at 465 nm, the longest wavelength used in the present study, photolyzed H_2O_2 resulting in the generation of DMPO-OH (Figure 5). The yield of DMPO-OH as a function of the wavelength was approximated to exponential curve while yield of DMPO-OH increased linearly with the concentration of H_2O_2 (Figure 7). On the other hand, LED irradiation of pure water and 1000 mM H_2O_2 without LED irradiation generated only a trace level of DMPO-OH (<0.25 μM). The two-way ANOVA revealed that the wavelength, the H_2O_2 concentration and their interaction significantly affected the yield of ·OH (Table 1).

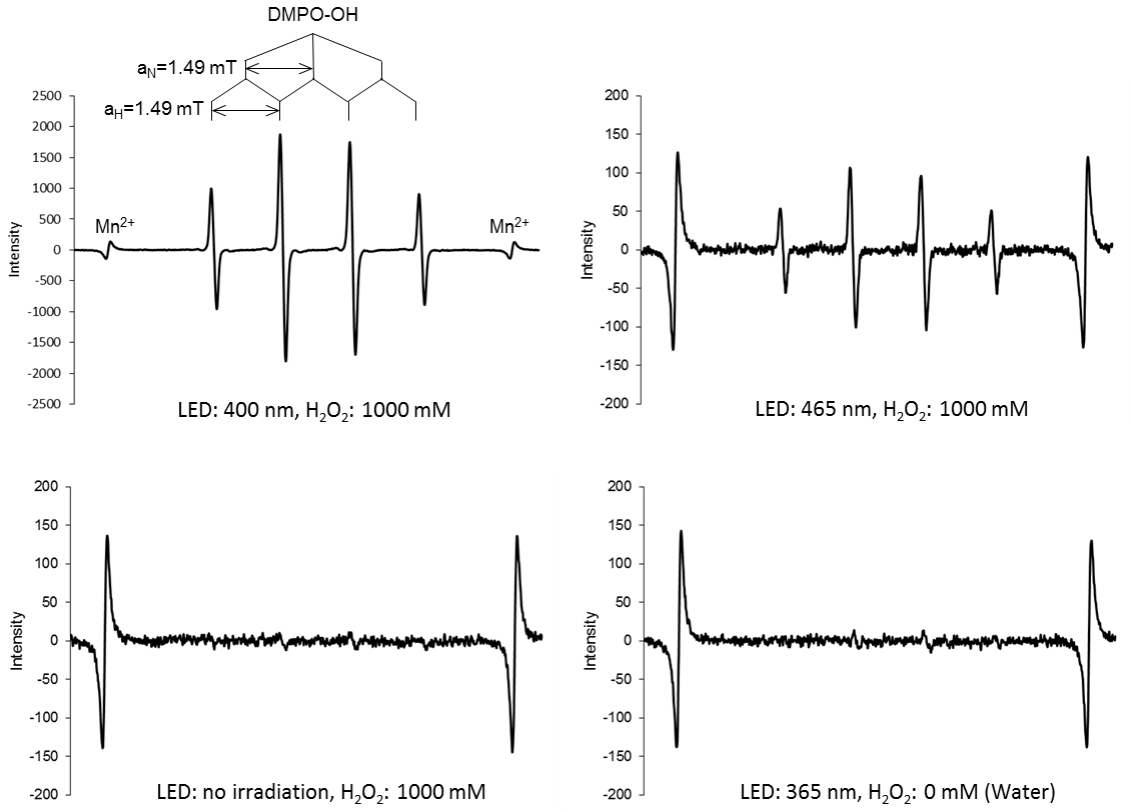

Figure 5. Representative ESR spectra of DMPO-OH

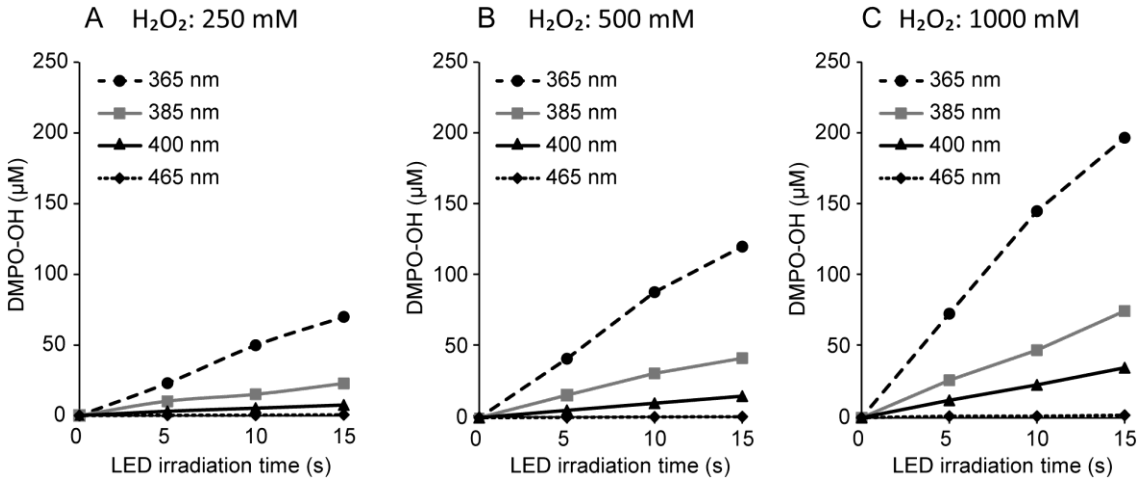

Figure 6. Yield of hydroxyl radicals trapped by DMPO (DMPO-OH). Each value represents the mean of triplicate measurements.

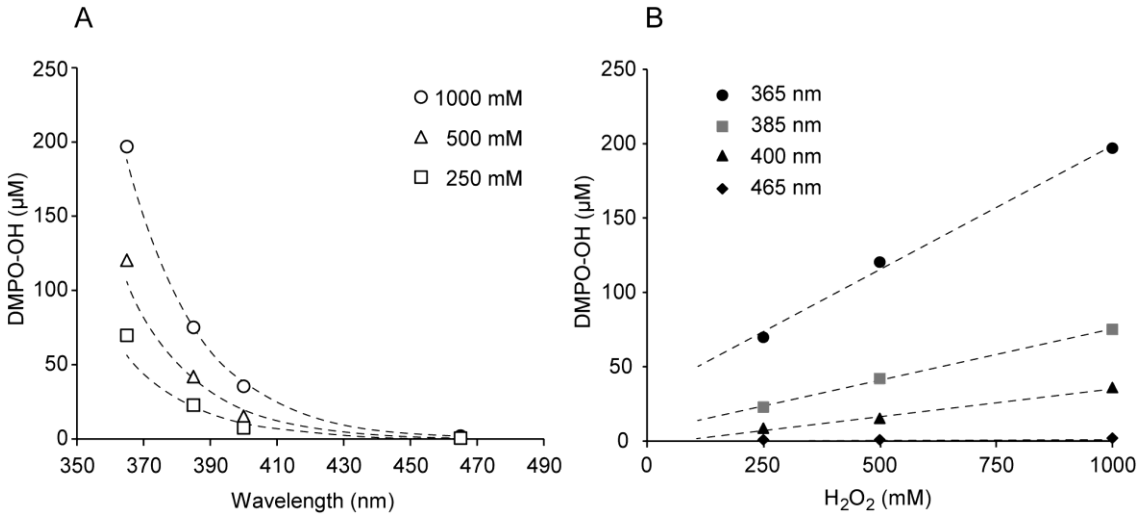

Figure 7. Effect of wavelength of LED (A) and concentration of H_2O_2 (B) on the yield of hydroxyl radicals. LED irradiation was performed for 15 s. Each value represents the mean of triplicate measurements.

Table 1. Summary table of two-way ANOVA for the changes in yield of hydroxyl radical

	Sum of squares	df	Mean square	F value	P value
WL	8734.35	3	2911.45	246.30	<0.0001
Conc.	24582.27	2	12291.14	1039.79	<0.0001
WL*Conc.	13346.24	6	2224.37	188.17	<0.0001
Error	283.70	24	11.82		

WL: wavelength, Conc.: concentration of H_2O_2, df: degree of freedom

Bactericidal assay. Figure 8 shows the results of bactericidal assay. When the bacterial suspension was mixed with H_2O_2 (H_2O_2: 250-1000 mM) followed by LED irradiation at wavelengths of 365, 385, 400 and 465 nm for 1 min, the CFU was significantly reduced.

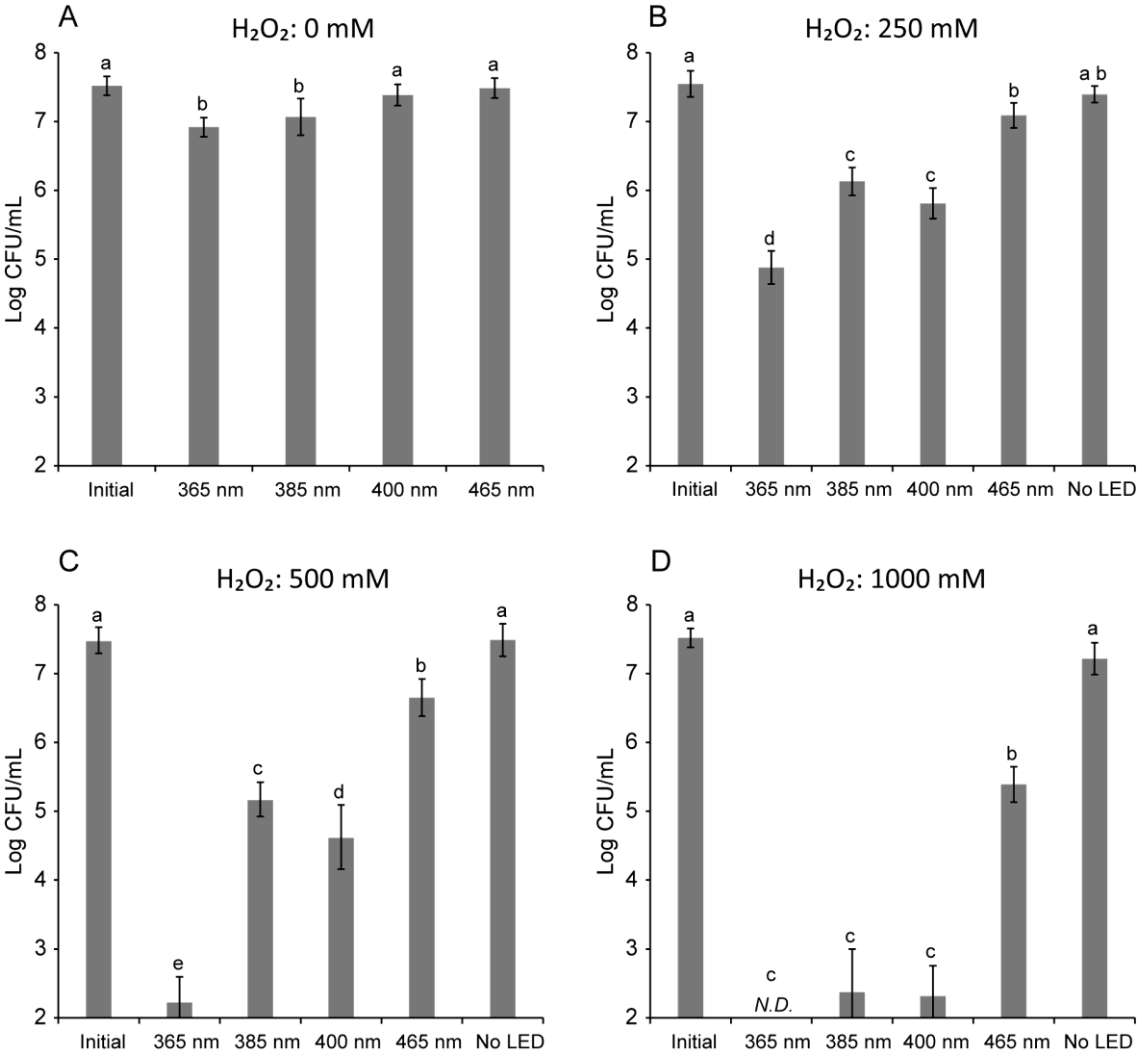

Figure 8. Influence of wavelength of LED and concentration of H_2O_2 on bactericidal activity of photolysis of H_2O_2 against *Staphylococcus aureus*. Each value represents the mean of sexuplicate measurements with standard deviation.

Different alphabetical letters above the columns show significant differences ($p<0.05$). N.D.: not detected.

Regarding the influence of factors on bactericidal activity, the reduction of CFU depended on the concentration of H_2O_2, and the LED irradiation of H_2O_2 at 365 nm showed highest bactericidal activity while the LED irradiation at 465 nm showed the lowest effect regardless of the concentration of H_2O_2. The bactericidal effects of the LED irradiations at 385 and 400 nm were in between those of irradiation at 365 and 465 nm. Almost the same bactericidal activity were observed in the irradiation at 385 and 400 nm except for the case that H_2O_2 was used at 500 mM where LED irradiation at 400 nm showed significantly higher bactericidal activity than the irradiation at 385 nm. In combination of the H_2O_2 concentration and the wavelength of light, irradiation of 500 mM H_2O_2 at 365 nm and of 1000 mM H_2O_2 at 365, 385 and 400 nm achieved bactericidal activity with a >5-log reduction of viable counts. By contrast to the photolysis of H_2O_2, the treatment with H_2O_2 alone (*i.e.* without LED irradiation) for 1 min did not show bactericidal effect even at the concentration of 1000 mM. When the bacterial suspension was mixed with pure water instead of H_2O_2, the LED irradiation at 365 and 385 nm for 1 min showed a mild bactericidal effect (*i.e.* less than 1-log

reduction of CFU/mL) but the irradiation at 400 and 465 nm did not.

The influence of the wavelength of light and the concentration of H_2O_2 on the bactericidal activity was statistically analyzed by the two-way ANOVA revealing that the wavelength, the concentration and the interaction of both significantly affected the bactericidal activity of the photolysis of H_2O_2 (Table 2).

Table 2. Summary table of two-way ANOVA for the changes in bactericidal activity of photolysis of H_2O_2 on *Staphylococcus aureus*

	Sum of squares	df	Mean square	F value	P value
WL	14.98	3	4.99	45.48	<0.0001
Conc.	30.78	2	15.39	140.20	<0.0001
WL*Conc.	18.27	6	3.05	27.74	<0.0001
Error	6.59	60	0.11		

WL: wavelength, Conc.: concentration of H_2O_2, df: degree of freedom

Discussion

In the present study, UVA and visible blue light were used for photolysis of H_2O_2. Based on the analysis of wavelength of LEDs, it was confirmed that the peak wavelengths were in accordance with those reported by the manufacturers. Although a difference was found in the FWHMs between OmniCure LX400+ and G-Light Prima-II, it is considered that the comparison of wavelength is possible because the peak of each spectrum is clearly distinguished.

When ESR spin trapping analysis is adopted to compare the yield of ·OH generated under different conditions, the treatment time-dependent generation of DMPO-OH should be confirmed because the level of DMPO-OH reaches plateau due to a methodological limitation of the analysis even though the ·OH is constantly generated (21). Thus, I firstly confirmed the time-dependent behavior, and then analyzed the effect of wavelength of light and the concentration of H_2O_2 on the yield of DMPO-OH. It was demonstrated that the yield of DMPO-OH as a function of the wavelength was approximated to an exponential curve. This would probably be attributable to the photon energy of light that is inversely proportion to the wavelength, resulting in more generation of ·OH via accelerated homolytic fission of H_2O_2 as the wavelength is shortened (13). Regarding the influence of H_2O_2 concentration, the yield of DMPO-OH

linearly increased with the concentration. This finding is in accordance with the previous study in our laboratory (1). In addition, the two-way ANOVA revealed that the wavelength and the concentration synergistically interacted in the generation of ·OH. Accordingly, when 250 mM H_2O_2 was irradiated with the 365 nm LED for 10 s, the yield of DMPO-OH (49.7±5.5 μM) was significantly higher than that generated by photolysis of 1000 mM H_2O_2 at 400 nm for 15 s (35.8±3.0 μM). In addition, as shown in Figure 7B, the shorter the wavelength was, the larger the slope of the regression line was (if the combination effect of H_2O_2 concentration and wavelength had been additive, the slopes would have become almost the same.).

Based on the ESR analysis, it was expected that the bactericidal effect of photolysis of H_2O_2 also depended on the wavelength of light as well as the concentration of H_2O_2. This was confirmed by the bactericidal assay. The LED irradiation of H_2O_2 at 365 and 465 nm displayed the highest and the lowest bactericidal activity, respectively, at any concentrations of H_2O_2. In addition, the bactericidal activities at 385 and 400 nm were in between those at 365 and 465 nm. However, there was an inconsistency between the bactericidal activities at 385 and 400 nm. Even though the yield of ·OH generated by the irradiation at 385 nm was higher than that by the irradiation at 400 nm, the

bactericidal activities were almost the same. One of the possibilities is the difference in the light penetration rate into bacterial cells. It is considered that the longer the wavelength is the deeper the light will penetrate as in the case of light penetration into the skin (14). Since the diffusion distance of ·OH is very limited because of its short-life (<10 ns) (23, 24), only ·OH generated in the vicinity of and/or inside the bacteria contributes to the bactericidal action. Thus, the light penetration rate could be one of the important factors which affect the bactericidal activity. Considering the bactericidal activities of photolysis at 365 and 385 nm, when the difference in the yields of ·OH was big enough, it would prevail the difference in the light penetration rates showing higher bactericidal activity. Indeed, the difference in the yield between 365 and 385 nm was about 140 μM when 1000 mM H_2O_2 was photolyzed for 15 s whilst that between 385 and 400 nm was about 30 μM. Similarly, although the yield of ·OH upon LED irradiation of 1000 mM H_2O_2 at 385 nm and that upon LED irradiation of 250 mM H_2O_2 at 365 nm were almost the same (Figure 7), the former bactericidal activity was much higher than the latter bactericidal activity (Figure 8). This inconsistency would be also attributable to the difference in the light penetration rate, which would in turn result in higher intracellular ·OH formation upon LED irradiation of 1000 mM H_2O_2 at 385 nm than that upon LED irradiation of 250 mM H_2O_2 at 365 nm. The underlying

mechanism of bactericidal activity dependently on the light penetration rate should further be studied. As for the influence of concentration of H_2O_2, the bacteria were killed with the increased concentration. This finding concurs with the previous study in our laboratory in which *E. faecalis* was killed by photolysis of H_2O_2 dependently on the concentration of H_2O_2 (1). The two-way ANOVA showed that the wavelength and the concentration also synergistically interacted in the bactericidal activity of photolysis of H_2O_2. Accordingly, the bactericidal effect of photolysis of 250 mM H_2O_2 at 365 nm was comparable to that of 500 mM H_2O_2 at 400 nm. Furthermore, although LED irradiation at 365 nm alone and 100 mM H_2O_2 alone exerted very weak and no bactericidal activity, respectively, the combination of the both resulted in more than 5-log reduction of viable cells.

By contrast to the photolysis of H_2O_2, the LED irradiation of water, even at 365 nm, generated ·OH only at a traceable level. This finding confirms that UVA does not deposit sufficient energy to cause photolysis of water, at least within the short-time (15 s) (13). In addition, the bactericidal effect of LED irradiation alone was limited (<1-log reduction of CFU) in comparison with that of the photolysis of H_2O_2. This would be due to the short treatment time of 1 min. If the treatment time is prolonged, the bactericidal

effect is likely enhanced as reported previously (25).

In the previous studies in our laboratory, it was shown that elevated temperature of H_2O_2 and addition of proanthocyanidin to H_2O_2 exerted a synergistic effect in a bactericidal activity of photolysis of H_2O_2 (2, 26). In addition to these synergistic effects, the present study demonstrated that the synergistic interaction of wavelength of light and concentration of H_2O_2 could also enhance the bactericidal activity of photolysis of H_2O_2. Therefore, by controlling these effects properly, it is possible to shorten the treatment time and/or to reduce the concentration of H_2O_2 without reduction in the bactericidal effect to be obtained with the method that was previously proposed in our laboratory (1). Besides the efficacy, we should further evaluate if shortened treatment time and reduced concentration of H_2O_2 brought by using UVA are more beneficial than using visible blue light in terms of safety aspect.

Conclusion

The present study clearly suggests that a combination of light-wavelength and H_2O_2 concentration could synergistically enhance the bactericidal activity of photolysis of H_2O_2. More in detail, by applying a light source emitting wavelengths less than 400 nm (especially UVA), it could be possible not only to shorten the treatment time but to reduce the H_2O_2 concentration. This means that the technique would be applied to disinfection in various fields such as medical devices and food processing, and to not only denture cleaning but the treatment of bacterial infectious diseases in dentistry once safety of the technique is verified.

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